Phosphopeptides

Significant differences exist in the specificity of phosphopeptides detected based on the method used for phosphopeptide enrichment (Fe IMAC, TIO2, etc) ...or do they?
Comprehensive and Reproducible Phosphopeptide Enrichment Using Iron Immobilized Metal Ion Affinity Chromatography (Fe-IMAC) Columns

Benjamin Ruprecht*, Heiner Koch*, Guillaume Medard*, Max Mundt*, Bernhard Kuster†‡∥, and Simone Lemeer†‡∥

Fig. 1. Workflow utilizing an Fe-IMAC HPLC column setup for the purification of phosphopeptides from complex digests. Fe-IMAC eluates can be either directly measured via LC-MS/MS, providing considerable throughput, or further fractionated via hSAX to provide increased analytical depth.
**Fig. 2. Fe-IMAC column characterization.**

A, Fe-IMAC UV chromatogram of 1 mg of A431 cell digest. The phosphopeptide fraction (reaction time: ~49 min) is well separated from the column breakthrough that contains the non-phosphorylated peptides. The inset on the left shows the number of phosphopeptides identified from the IMAC eluate and the number of phosphopeptides identified from a second IMAC enrichment of the column flow-through (FT) of the first Fe-IMAC enrichment. The inset on the right shows the respective UV traces. Both indicate that the A431 digest was essentially depleted of phosphopeptides. B, UV signal-based quantification of Fe-IMAC enriched phosphopeptides as a function of the amount of cellular digest applied. It is apparent that the column captured phosphopeptides over a wide linear range. C, Venn diagram of the number of unique phosphopeptide identifications across three technical replicates of Fe-IMAC purification of phosphopeptides from 1 mg of A431 lysate digest.
<table>
<thead>
<tr>
<th></th>
<th>Fe-IMAC column</th>
<th>Fe-IMAC column</th>
<th>Fe-IMAC column</th>
<th>Ti-IMAC tip</th>
<th>Ti-IMAC tip</th>
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Take home message:

- Apparent Complementarity of Phosphopeptide Enrichment Methods Is Primarily Caused by Inefficient Elution and Insufficient Mass Spectrometric Capacity
High pH reverse phase is a good place to start for phosphopeptide enrichment
Table 1. High-pH Reversed-Phase (HpH) and Strong Cation Exchange (SCX) Phosphopeptide Variants Comparison Based on Four Biological Replicates for Each

<table>
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<th>fractionation</th>
<th>1 P</th>
<th>2 P</th>
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<th>non-phoso</th>
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<td>HpH</td>
<td>12 609 (±2655)</td>
<td>4199 (±1005)</td>
<td>759 (±165)</td>
<td>17 566 (±3737)</td>
<td>892 (±256)</td>
<td>95 (±2)</td>
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<tr>
<td>SCX</td>
<td>1561 (±330)</td>
<td>3360 (±1077)</td>
<td>1295 (±519)</td>
<td>6215 (±1759)</td>
<td>350 (±287)</td>
<td>94 (±6)</td>
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Figure 3. (A) Diagram demonstrating optimal range for MS parameters depending on phosphoproteomics sample complexity. The colored boxes represent the ideal working area. (B) Box plot of Andromeda score distribution for phosphopeptides detected in the three different experiments.

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Phosphopeptide Enrichment

In-solution phosphopeptide enrichment using PolyMAC-Ti

Mixture of peptides  PolyMAC  Solid-phase beads

150 ug Cell Lysate + 150ug Super Silac MM standard
~1100 Proteins
~2200 phosphopeptides
  1232 single phosphorylation  pS  84%
  671 double phosphorylated  pT  15%
  67 with 3 or 4 phosphorylation sites  pY  1%
2400 phosphorylation sites

MAPK3(ERK1) & MAPK1(ERK2)

http://tymora-analytical.com/products/polymac/
Super-SILAC
Spike-in

Labelled cell lines are combined and spiked into unlabeled samples

Multiple samples are compared via comparison to the labelled standard

(A) Murine brain and yeast lysates were digested and phosphopeptides enriched by TiO2. The mouse phosphopeptides were labeled with TMT and mixed at a relative concentration of 10:2:1:1:2:10. Yeast phosphopeptides were split into three samples, labeled with three TMT reagents, and mixed 10:10:10:0:0:0. The mouse and yeast phosphopeptides were mixed (1:1, w:w). If present, interference from the yeast background would perturb mouse ratios. For all mouse phosphopeptides, the TMT channels lacking any yeast interference provided control intensities and ratios. (B) SPS-MS3 method overview. Synchronous precursor selection (SPS) enables the simultaneous isolation of multiple MS2 fragment ions increasing TMT reporter ion signal in the MS3 scan.
Phosphopeptide mixture was analyzed by LC–MS and quantified by MS2 and SPS-MS3 methods. SPS-MS3 performance was further investigated by varying the number of MS2 fragment ions included in the quantitative MS3 spectrum (i.e., 1, 3, 6, and 10 precursor ions). (A–E) Distributions of ratios corresponding to yeast phosphopeptides (TMT channels 126/127, green trace), mouse phosphopeptides with interference (TMT channels 126/127, red trace), and mouse phosphopeptides without interference (TMT channels 131/130, blue trace). Yeast phosphopeptides (green) are expected at a 1:1 ratio while the mouse phosphopeptides were mixed at a 5:1 ratio (red and blue). The dashed line depicts the expected ratio of 5:1. The number of quantified mouse phosphopeptides is displayed for each method. Quantification via an MS2 method (A) resulted in significant ratio distortion with a wide distribution of ratios. Utilizing a MS3 method (B–E) dramatically improves the accuracy and precision (fwhm) of phosphopeptide quantification.
(A) 10-plex TMT phosphopeptide preparation. Following proteolytic digestion, phosphopeptides were enriched by TiO2 and labeled with the 10-plex TMT reagents. Subsequent offline, basic pH reversed-phase fractionation was employed. (B) The instrument interrogated each sample using a data-dependent, SPS-MS3 method. Following ITMS2 analysis of the precursor ions, up to 10 MS2 fragments (light gray bars) were isolated and further fragmented to provide the quantitative MS3 spectrum. Reporter ion intensities corresponding to the 10 TMT channels were normalized, scaled, and summarized for the five brain and liver replicates.
A good place to start for an enrichment protocol

Rapid and Reproducible Single-Stage Phosphopeptide Enrichment of Complex Peptide Mixtures: Application to General and PhosphoTyrosine-Specific Phosphoproteomics Experiments

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Supporting Information

ABSTRACT: Reversible protein phosphorylation is an essential regulatory component of virtually every cellular process and is frequently dysregulated in cancer. However, significant analytical barriers persist that hamper the routine application of phosphoproteomics in translational settings. Here, we present a straightforward and reproducible approach for the broad-scale analysis of protein phosphorylation that relies on a single phosphopeptide enrichment step using titanium dioxide microspheres from whole cell lysate digests and compared it to the well-established SCX-TiO₂ workflow for phosphopeptide purification on a proteome-wide scale. We demonstrate the scalability of our approach from 200 μg to 5 mg of total NCI-H23 non-small cell lung adenocarcinoma cell lysate digest and determine its quantitative reproducibility by label-free analysis of phosphopeptide peak areas from replicate purifications (median CV: 20% RSD). Finally, we combine this approach with immunoaffinity phosphotyrosine enrichment, enabling the identification of 3168 unique nonredundant phosphotyrosine peptides in two LC-MS/MS runs from 8 mg of HeLa peptides, each with 80% phosphotyrosine selectivity, at a peptide FDR of 0.2%. Taken together, we establish and validate a robust approach for proteome-wide phosphorylation analysis in a variety of scenarios that is easy to implement in biomedical research and translational settings.